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<b>(21) International Application Number:</b> PCT/DK96/00031 <b>(22) International Filing Date:</b> 19 January 1996 (19.01.96)  <b>(30) Priority Data:</b> 0061/95 19 January 1995 (19.01.95) DK  <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S-[DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> BECH, Lisbeth [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). RASMUSSEN, Grethe [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). HALKIER, Torben [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). OKADA, Mariko [JP/JP]; Novo Nordisk Bioindustry Ltd., Makuhari Techno Garden CB-6, 3, Nakase 1-chome, Chiba-shi 261-01 (JP). ANDERSEN, Lene, Nonboe [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). KAUPPINEN, Markus, Sakari [FI/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). SANDAL, Thomas [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK).		<b>(74) Common Representative:</b> NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).  <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> With international search report.
<b>(54) Title:</b> TRANSGLUTAMINASES FROM OOMYCETES  <b>(57) Abstract</b> <p>Transglutaminase and transglutaminase preparations can be produced by lower fungi belonging to the class <i>Oomycetes</i> and unprecedented high-level expression is achievable by growing these coenocytium forming organisms, especially the strains <i>Pythium</i> sp., <i>Pythium irregulare</i>, <i>Pythium dissotocum</i>, <i>Pythium perillium</i> (or <i>P. periplocum</i>), <i>Pythium torulosum</i>, <i>Pythium ultimum</i>, <i>Pythium aphanidermatum</i>, <i>Phytophthora cactorum</i>, <i>Phytophthora palmivora</i>, <i>Phytophthora porri</i>, <i>Phytophthora infestans</i>, <i>Phytophthora megasperma</i>, <i>Phytophthora cinnamomi</i> and <i>Phytophthora cryptogea</i>; and a recombinant transglutaminase has been cloned and expressed, the enzyme and enzyme preparations being useful for cross-linking proteins, e.g. in flour, baked products, meat products, fish products, cosmetics, cheese, milk products, gelled food products and leather finishing, or as a glutaminase, e.g. in bread and other baked gluten-containing food products.</p>		

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## TRANSGLUTAMINASES FROM OOMYCETES

The present invention relates to novel transglutaminase preparations derivable from the class *Oomycetes*, a novel  
5 transglutaminase derived from *Phytophthora cactorum*, CBS 618.94 or IFO 30474, a DNA construct encoding the transglutaminase enzyme, a method of producing the novel transglutaminase and the novel transglutaminase preparation, a method for producing a gel or protein gelation  
10 composition, and the use thereof.

## BACKGROUND OF THE INVENTION

15 Transglutaminases are enzymes capable of catalyzing an acyl transfer reaction in which a gamma-carboxyamide group of a peptide-bound glutamine residue is the acyl donor. Primary amino groups in a variety of compounds may function as acyl acceptors with the subsequent formation  
20 of monosubstituted gamma-amides of peptide-bound glutamic acid. When the  $\epsilon$ -amino group of a lysine residue in a peptide-chain serves as the acyl acceptor, the transglutaminases form intramolecular or intermolecular  $\epsilon$ -( $\gamma$ -Glu)-Lys crosslinks.

25

This peptide crosslinking activity is useful for a variety of industrial purposes, including gelling of proteins, reduction of antigenicity of proteins, improvement of baking quality of flour, producing paste type food  
30 materia from protein, fat and water, preparation of cheese from milk concentrate, binding of chopped meat product, improvement of taste and texture of food proteins, producing jelly, gel cosmetics etc.

35 A wide array of transglutaminases have been isolated and characterized from animals and plants. The animal derived TGases are  $\text{Ca}^{2+}$ -dependent and often multi-subunit enzymes. The most widely used mammalian transglutaminase, FXIIIa, is product inhibited, difficult to obtain in high

amounts and thus expensive, and therefore not useful, for all applications.

A few microbial TGases have been described, including the  
5  $\text{Ca}^{2+}$ -independent TGases from *Streptomyces* disclosed  
in US 5,156,956 and related species disclosed in US  
5,252,469.

The yields of the microbial transglutaminases in shake-  
10 flasks and fermentors are far below those seen for other  
industrial enzymes. Thus, better production methods, in-  
cluding new high-yielding producers are needed.  
Previously, this goal has been pursued by applying con-  
ventional recombinant DNA techniques for cloning and ex-  
15 pression in *E. coli*, *S. cerevisiae* and *S. lividans*  
(Washizu et al.; Tahakana et al.; Takagi et al.) but wit-  
hout success.

Klein et al. found and partially characterized a trans-  
20 glutaminase from the slime mold *Physarum polycephalum*  
which is a homodimer having a total molecular weight of  
77 kDa. JP 6078783 Kokai relates to the use of this  
transglutaminase for protein gelation. However, it is  
well-known that slime molds are unsuited for large-scale  
25 industrial fermentation. Further, *Physarum* is not a fun-  
gus; it belongs to the *Myxomycetes* (Entrez NIH data base,  
current version January 1996). Taxonomically, the only  
common feature of *Oomycetes*, *Myxomycetes* and *Eumycota*  
(fungi) is that they all are mitochondrial eukaryotes.

30

The object of the invention is to provide a novel trans-  
glutaminase, a novel transglutaminase preparation, a met-  
hod for producing the transglutaminase or transglutamina-  
se preparation in a better yield and higher purity than  
35 hitherto possible which transglutaminase can be used eit-  
her alone or in combination with other enzymes for indu-  
strial purposes.

## SUMMARY OF THE INVENTION

Surprisingly, it has been found that organisms belonging to the class *Oomycetes* produce transglutaminase and that  
5 high-level expression is achievable by growing these coenocytium forming organisms.

In particular, isolates belonging to the class *Oomycetes* have been shown to express transglutaminases in unprecedented high amounts, including isolates belonging to the  
10 order *Peronosporales*, family *Pythiaceae*, and the genera *Pythium* and *Phytophthora*.

Accordingly, the present invention relates to transglutaminase preparations producible by cultivation of a transglutaminase producing strain of the class *Oomycetes* and to novel transglutaminases derived from transglutaminase producing strains of the class *Oomycetes*. Preferably, the novel transglutaminase and the transglutaminase preparation of the invention are derived from or producible by  
15 20 transglutaminase producing strains belonging to the class *Oomycetes*.

Further, the present invention relates to a parent transglutaminase derived from or producible by a species selected from *Phytophthora cactorum*, CBS 618.94 or IFO 30474, *Phytophthora cryptogea*, CBS 651.94, *Pythium periplum* (or *P. periplocum*), CBS 620.94, *Pythium irregulare*, CBS 701.95, *Pythium sp.*, CBS 702.95, *Pythium intermedium*,  
25 30 CBS 703.95, *Pythium sp.*, CBS 704.95, *Pythium ultimum*, CBS 705.95 or a functional analogue thereof.

The present invention also relates to a method for the production of a transglutaminase preparation according to the invention by cultivating, in a suitable medium, a  
35 strain belonging to the class *Oomycetes*, preferably belonging to an order selected from *Peronosporales*, *Saprolegniales*, *Leptomitales* and *Lagenidiales*, more preferably belonging to a family selected from *Pythiaceae*, *Perono-*

"sporaceae, Saprolegniaceae, Leptomitaceae, Rhiphidiaceae, and Lagenidiaceae, especially belonging to a genus selected from *Pythium* and *Phytophthora*.

5 Further, the present inventors have now surprisingly succeeded in isolating and characterizing a DNA sequence from a strain of the oomycetes *Phytophthora cactorum* exhibiting transglutaminase activity, thereby making it possible to prepare a recombinant transglutaminase.

10

Accordingly, in yet another aspect the invention relates to a DNA construct comprising a DNA sequence encoding an enzyme exhibiting transglutaminase activity, which DNA sequence comprises

15

a) the DNA sequence shown in SEQ ID No. 1, and/or the DNA sequence obtainable from the plasmid in *Escherichia coli* DSM 10256 or

20 b) an analogue of the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from the plasmid in *Escherichia coli* DSM 10256, which

i) is homologous with the DNA sequence shown in SEQ ID  
25 No. 1 and/or the DNA sequence obtainable from the plasmid in *Escherichia coli* DSM 10256, or

ii) hybridizes with the same oligonucleotide probe as the DNA sequence shown in SEQ ID No. 1 and/or the DNA  
30 sequence obtainable from the plasmid in *Escherichia coli* DSM 10256, or

iii) encodes a polypeptide which is homologous with the polypeptide encoded by a DNA sequence comprising  
35 the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from the plasmid in *Escherichia coli* DSM 10256, or

iv) encodes a polypeptide which is immunologically

reactive with an antibody raised against the purified transglutaminase encoded by the DNA sequence shown in SEQ ID No 1 and/or the DNA sequence obtainable from the plasmid in *Escherichia coli* DSM 10256.

5

It is believed that the DNA sequence shown in SEQ ID No. 1 is identical to the DNA sequence obtainable from the plasmid in *Escherichia coli* DSM 10256.

- 10 The strain *Escherichia coli* was deposited under the deposition number DSM 10256 on 18 September 1995 at the DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Maascheroder Weg 1b, D-38125 Braunschweig, Germany, according to the Budapest Treaty.

15

In another aspect, the invention relates to a method of crosslinking proteins comprising contacting a proteinaceous substrate with a transglutaminase or transglutaminase preparation of the present invention.

20

In yet another aspect, the invention relates to use of the transglutaminase or transglutaminase preparation of the present invention in flour, baked products, meat products, fish products, cosmetics, cheese, milk products, 25 gelled food products and leather finishing.

#### DETAILED DESCRIPTION OF THE INVENTION

- 30 In the present specification and claims, the term "transglutaminase" is intended to be understood as an enzyme capable of catalyzing an acyl transfer reaction in which a gamma-carboxyamide group of a peptide-bound glutamine residue is the acyl donor.

35

In the present context the term "derivable" or "derived from" is intended not only to indicate a transglutaminase produced by a strain of the organism in question, but also a transglutaminase encoded by a DNA sequence isola-

ted from such strain and produced in a host organism, transformed with said DNA sequence. Furthermore, the term is intended to indicate a transglutaminase which is encoded by a DNA sequence of synthetic and/or cDNA origin and  
5 which has the identifying characteristics of the transglutaminase in question.

The transglutaminase may be a component occurring in an enzyme system produced by a given microorganism, such an  
10 enzyme system mostly comprising several different enzyme components. In the present specification and claims, such an enzyme system comprising at least one transglutaminase component is denoted "transglutaminase preparation".

15 Alternatively, the transglutaminase may be a single component, i.e. a component essentially free of other enzyme components usually occurring in an enzyme system produced by a given microorganism, the single component being a recombinant component, i.e. produced by cloning of a DNA  
20 sequence encoding the single component and subsequent cell transformed with the DNA sequence and expressed in a host. The host is preferably a heterologous host, but the host may under certain conditions also be the homologous host. A recombinant transglutaminase may be cloned and  
25 expressed according to standard techniques conventional to the skilled person.

According to the present invention, the native or unmodified transglutaminase is of microbial origin, more specifically obtainable from a strain belonging to the class  
30 *Oomycetes*.

The class *Oomycetes* comprises the orders *Peronosporales*, *Saprolegniales*, *Leptomitales* and *Lagenidiales*.

35

The order *Peronosporales* comprises the families *Pythiaceae*, *Peronosporaceae*, *Peronophytophthoraceae* and *Albuginaceae*.



The order *Saprolegniales* comprises the families *Saprolegniaceae*, *Ectrogellaceae*, *Thraustochytriaceae*, *Haliphthoraceae* and *Leptolegniellaceae*.

- 5 The order *Leptomitales* comprises the families *Leptomitaceae* and *Rhiphidiaceae*.

The order *Lagenidiales* comprises the families *Lagenidiaceae*, *Olpidiaceae* and *Sirolpidiaceae*.

10

- It is contemplated that all orders and all families taxonomically belonging to the class *Oomycetes* comprise transglutaminase producing strains. In this respect it should be noted that the families *Peronophytophthoraceae*,  
15 *Albuginaceae*, *Ectrogellaceae*, *Thraustochytriaceae*, *Haliphthoraceae*, *Leptolegniellaceae*, *Olpidiaceae* and *Sirolpidiaceae* are small and often highly specialised. Thus, the families *Pythiaceae*, *Peronosporaceae*, *Saprolegniaceae*, *Leptomitaceae*, *Rhiphidiaceae* and *Lagenidiaceae* should  
20 be considered as being representative of the *Oomycetes*.

- In a preferred embodiment, the transglutaminase preparation of the present invention is producible by a transglutaminase producing strain which taxonomically belongs  
25 to the family *Pythiaceae*, preferably to the genus *Pythium* or the genus *Phytophthora*, more preferably to a subdivision of the genus *Pythium* Pringsheim (Waterhouse) or a subdivision of the genus *Phytophthora* deBary (Newhook, Waterhouse and Stamps). In the following, examples of  
30 members of all subdivisions (I-III) of genus *Pythium*, and all subdivisions (I-VI) of genus *Phytophthora* are given. Examples of transglutaminase producing species of the genus *Pythium* are

- I) *P. irregulare*, CBS 701.95;  
35 IIA<sub>1</sub>) *P. dissotocum*;  
IIA<sub>2</sub>) *P. periillum* (or *P. periplocum*); *P. torulosum*; *P. aphanidermatum*; preferably *P. periillum* (or *P. periplocum*), CBS 620.94;  
IIB) *P. ultimum*, CBS 705.95;

III) *P.intermedium*, CBS 703.95.

Examples of transglutaminase producing species of the genus *Phytophthora* are

- I) *P. cactorum*; preferably *P. cactorum*, CBS 618.94 and  
5 IFO 30474.
- II) *P.palmivora*;
- III) *P.porri*;
- IV) *P. infestans*;
- V) *P.megasperma*;
- 10 VI) *P. cryptogea*; and *P. cinnamomi*; preferably *P. crypto-*  
*gea*, CBS 651.94.

In another preferred embodiment, the transglutaminase preparation of the present invention is producible by a  
15 transglutaminase producing strain which taxonomically belongs to the family *Peronosporaceae*, preferably to the genus *Plasmopara*, more preferably to the species *Plasmopara halstedii*.

20 In yet another preferred embodiment, the transglutaminase preparation of the present invention is producible by a transglutaminase producing strain which taxonomically belongs to the family *Saprolegniaceae*, preferably to a genus selected from the genera *Achlya*, *Saprolegnia* and  
25 *Aphanomyces*.

In yet another preferred embodiment, the transglutaminase preparation of the present invention is producible by a transglutaminase producing strain which taxonomically  
30 belongs to the family *Leptomitaceae*, preferably to a genus selected from the genera *Apodachlya* and *Leptomitus*.

In yet another preferred embodiment, the transglutaminase preparation of the present invention is producible by a  
35 transglutaminase producing strain which taxonomically belongs to the family *Rhiphidiaceae*, preferably to a genus selected from the genera *Aqualinderella* and *Rhiphidium*.

In yet another preferred embodiment, the transglutaminase preparation of the present invention is producible by a transglutaminase producing strain which taxonomically belongs to the family *Lagenidiaceae*, preferably to a genus selected from the genera *Lagenidium* and *Olpidiopsis*.

In a preferred aspect of the invention, it is contemplated that novel transglutaminases are obtainable by or derivable from species selected from the group of genera consisting of *Pythium* and *Phytophthora*, more preferably from the species *Pythium periilum* (or *P. periplocum*), *Pythium irregulare*, *Pythium sp.*, *Pythium ultimum*, *Pythium intermedium*, *Phytophthora cactorum* and *Phytophthora cryptogea*, especially from the species *Pythium periilum* (or *P. periplocum*) deposited at Centraalbureau voor Schimmelcultures, Oosterstraat 1, NL-3742 SK Baarn, The Netherlands on December 20, 1994 under the deposition number CBS 620.94; *Phytophthora cactorum* deposited at Centraalbureau voor Schimmelcultures under the deposition number CBS 618.94 on December 20, 1994 (and redeposited on 19 October, 1995) and previously at the Institute for Fermentation, Osaka, under the deposition number IFO 30474; *Phytophthora cryptogea* deposited at Centraalbureau voor Schimmelcultures on December 27, 1994 under the deposition number CBS 651.94; *Pythium irregulare* deposited at Centraalbureau voor Schimmelcultures on 19 October, 1995 under the deposition number CBS 701.95; *Pythium sp.* deposited at Centraalbureau voor Schimmelcultures on 19 October, 1995 under the deposition number CBS 702.95; *Pythium intermedium* deposited at Centraalbureau voor Schimmelcultures on 19 October, 1995 under the deposition number CBS 703.95; *Pythium sp.* deposited at Centraalbureau voor Schimmelcultures on 19 October, 1995 under the deposition number CBS 704.95; *Pythium ultimum* deposited at Centraalbureau voor Schimmelcultures on 19 October, 1995 under the deposition number CBS 705.95; all depositions made under the Budapest Treaty.

The transglutaminase component may be derived either from

the homologous or a heterologous host. Preferably, the component is homologous. However, a heterologous component which is immunologically reactive with an antibody raised against a highly purified transglutaminase and  
5 which is derived from a specific microorganism is also preferred.

Advantageously, a parent transglutaminase derivable from a strain of the genera *Pythium* and *Phytophthora* may be  
10 used.

In a preferred embodiment, the parent transglutaminase is selected from the group consisting of a *Phytophthora cactorum*, CBS 618.94/IFO 30474, transglutaminase; a *Pythium*  
15 *perillium* (or *P. periplocum*), CBS 620.94, transglutaminase; a *Pythium irregulare*, CBS 701.95, transglutaminase; a *Pythium* sp., CBS 702.95, transglutaminase; a *Pythium intermedium*, CBS 703.95, transglutaminase; a *Pythium* sp., CBS 704.95, transglutaminase; a *Pythium ultimum*, CBS  
20 705.95, transglutaminase and a *Phytophthora cryptogea*, CBS 651.94, transglutaminase; or is a functional analogue of any of said parent transglutaminases which

(i) comprises an amino acid sequence being at least 40%,  
25 preferably at least 60%, especially more than 74%, homologous with the amino acid sequence of the parent transglutaminase,

(ii) reacts with an antibody raised against the parent  
30 transglutaminase, and/or

(iii) is encoded by a DNA sequence which hybridizes with the same probe as a DNA sequence encoding the parent transglutaminase.

35

Property i) of the analogue is intended to indicate the degree of identity between the analogue and the parent transglutaminase indicating a derivation of the first sequence from the second. In particular, a polypeptide is

considered to be homologous to the parent transglutaminase if a comparison of the respective amino acid sequences reveals an identity of greater than about 40%, such as above 45%, 50%, 55%, 60%, 65%, 70%, 74%, 80%, 85%, 90% or even 95%. Sequence comparisons can be performed via known algorithms, such as the one described by Lipman and Pearson (1985).

The additional properties ii) and iii) of the analogue of the parent transglutaminase may be determined as follows:

Property ii), i.e. the immunological cross reactivity, may be assayed using an antibody raised against or reactive with at least one epitope of the parent transglutaminase. The antibody, which may either be monoclonal or polyclonal, may be produced by methods known in the art, e.g. as described by Hudson et al., 1989. The immunological cross-reactivity may be determined using assays known in the art, examples of which are Western Blotting or radial immunodiffusion assay, e.g. as described by Hudson et al., 1989.

The probe used in the characterization of the analogue in accordance with property iii) defined above, may suitably be prepared on the basis of the full or partial nucleotide or amino acid sequence of the parent transglutaminase. The hybridization may be carried out under any suitable conditions allowing the DNA sequences to hybridize. For instance, such conditions are hybridization under specified conditions, e.g. involving presoaking in 5xSSC and prehybridizing for 1h at -45°C in a solution of 5xSSC, 5xDenhardt's solution, 0.5% SDS, and 100 µg/ml of denatured sonicated salmon sperm DNA, followed by hybridization in the same solution supplemented with <sup>32</sup>P-dCTP-labelled probe for 12h at -45°C, or other methods described by e.g. Sambrook et al., 1989.

In the present context, the "analogue" of the DNA sequence shown in SEQ ID No. 1 is intended to indicate any DNA

sequence encoding an enzyme exhibiting transglutaminase activity, which has any or all of the properties i)-iv) of claim 27. The analogous DNA sequence

5 a) may be isolated from another or related (e.g. the same) organism producing the enzyme with transglutaminase activity on the basis of the DNA sequence shown in SEQ ID No. 1, e.g. using the procedures described herein, and thus, e.g. be an allelic or species variant of the DNA  
10 sequence comprising the DNA sequences shown herein,

b) may be constructed on the basis of the DNA sequence shown in SEQ ID No. 1, e.g. by introduction of nucleotide substitutions which do not give rise to another amino  
15 acid sequence of the transglutaminase encoded by the DNA sequence, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence. However, in  
20 the latter case amino acid changes are preferably of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-  
25 terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain. See in general Ford et al., Protein Expression and Purification 2: 95-107, 1991.  
30

Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine).

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acids essential to the activity of the polypeptide encoded by the DNA construct of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244, 1081-1085, 1989). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological (i.e. transglutaminase) activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance, crystallography or photoaffinity labeling. See, for example, de Vos et al., Science 255: 306-312, 1992; Smith et al., J. Mol. Biol. 224: 899-904, 1992; Wlodaver et al., FEBS Lett. 309: 59-64, 1992.

The homology referred to in i) above or of claim 27 is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., Journal of Molecular Biology, 48: 443-453, 1970). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the DNA sequence exhibits a degree of identity preferably of at least 40%, more preferably at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 74%, even more preferably at least 80%, especially at least 90%, with the coding region of the DNA sequence shown in SEQ ID No.1.

The hybridization referred to in ii) above or of claim 27, is intended to indicate that the analogous DNA sequence hybridizes to the same probe as the DNA sequence encoding the transglutaminase enzyme under certain specified conditions which are described in detail in the Materials and Methods section hereinafter. Normally, the analogous DNA sequence is highly homologous to the DNA sequence such as at least 70% homologous to the DNA sequence shown in SEQ ID No. 1 encoding an transglutaminase of the invention, such as at least 75%, at least 80%, at least 85%, at least 90% or even at least 95% homologous to said DNA sequence.

The homology referred to in iii) above or of claim 27 is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., *Journal of Molecular Biology*, 48: 443-453, 1970). Using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1, the polypeptide encoded by a homologous DNA sequence exhibits a degree of identity preferably of at least 70%, more preferably at least 75%, most preferably at least 80%, especially at least 90%, with the enzyme encoded by a DNA construct comprising the DNA sequence shown in SEQ ID No.1.

In connection with property iv) above or of claim 27 it is intended to indicate a transglutaminase encoded by a DNA sequence isolated from strain CBS 618.94 and produced in a host organism transformed with said DNA sequence or produced by the strain CBS 618.94. The immunological reactivity may be determined by the method described in the Materials and Methods section below.

In further aspects the invention relates to an expression vector harbouring a DNA construct of the invention, a



cell comprising the DNA construct or expression vector  
and a method of producing an enzyme exhibiting transglu-  
taminase activity which method comprises culturing said  
cell under conditions permitting the production of the  
5 enzyme, and recovering the enzyme from the culture.

In a still further aspect the invention relates to an  
enzyme exhibiting transglutaminase activity, which enzyme

- 10 a) is encoded by a DNA construct of the invention  
b) produced by the method of the invention, and/or  
c) is immunologically reactive with an antibody raised  
against a purified transglutaminase encoded by the DNA  
sequence shown in SEQ ID No.1.

15

The transglutaminase mentioned in c) above may be encoded  
by the DNA sequence isolated from the strain *Phytophthora*  
*cactorum*, CBS 618.94, and produced in a host organism  
transformed with said DNA sequence or produced by the  
20 strain CBS 618.94.

The DNA sequence of the invention encoding an enzyme ex-  
hibiting transglutaminase activity may be isolated by a  
general method involving

- 25 - cloning, in suitable vectors, a DNA library from *Phy-*  
*tophthora cactorum*,  
- transforming suitable yeast host cells with said vec-  
tors,  
- culturing the host cells under suitable conditions to  
30 express any enzyme of interest encoded by a clone in  
the DNA library,  
- screening for positive clones by determining any  
transglutaminase activity of the enzyme produced by  
such clones, and  
35 - isolating the enzyme encoding DNA from such clones.

The general method is further disclosed in WO 94/14953  
the contents of which are hereby incorporated by referen-  
ce. A more detailed description of the screening method

is given in Example 5 below.

The DNA sequence coding for the enzyme may for instance be isolated by screening a cDNA library of *Phytophthora cactorum*, and selecting for clones expressing transglutaminase activity, or from *Escherichia coli*, DSM 10256. The appropriate DNA sequence may then be isolated from the clone by standard procedures, e.g. as described in Example 5.

10

It is expected that a DNA sequence coding for a homologous enzyme, i.e. an analogous DNA sequence, is obtainable from other microorganisms. For instance, the DNA sequence may be derived by similarly screening a cDNA library of another fungus, such as a strain of *Pythium*.

15

Alternatively, the DNA coding for a transglutaminase of the invention may, in accordance with well-known procedures, conveniently be isolated from DNA from a suitable source, such as any of the above mentioned organisms, by use of synthetic oligonucleotide probes prepared on the basis of a DNA sequence disclosed herein. For instance, a suitable oligonucleotide probe may be prepared on the basis of the nucleotide sequence shown in SEQ ID No. 1 or any suitable subsequence thereof.

20

The DNA sequence may subsequently be inserted into a recombinant expression vector. This may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

30

35

In the vector, the DNA sequence encoding the transglutaminase should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the transglutaminase, the promoter and the terminator, respectively, and to insert them into suitable vectors are well known to persons skilled in the art (cf., for instance, Sambrook et al., 1989).

The host cell which is transformed with the DNA sequence encoding the enzyme of the invention is preferably a eukaryotic cell, in particular a fungal cell such as a yeast or filamentous fungal cell. In particular, the cell may belong to a species of *Aspergillus* or *Trichoderma*, most preferably *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of *Aspergillus* as a host microorganism is described in EP 238 023 (of Novo Nordisk A/S), the contents of which are hereby incorporated by reference. The host cell may also be a yeast cell, e.g. a strain of *Saccharomyces*, in particular *Saccharomyces cerevisiae*, *Saccharomyces kluyveri* or *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* sp., such as *Schizosaccharomyces pombe*, a strain of *Hansenula* sp. *Pichia* sp., *Yarrowia* sp. such as *Yarrowia lipolytica*, or *Kluyveromyces* sp. such as *Kluyveromyces lactis*.

In a still further aspect, the present invention relates to a method of producing an enzyme according to the invention, wherein a suitable host cell transformed with a DNA sequence encoding the enzyme is cultured under conditions permitting the production of the enzyme, and the resulting enzyme is recovered from the culture.

The medium used to culture the transformed host cells may, be any conventional medium suitable for growing the host cells in question. The expressed transglutaminase may conveniently be secreted into the culture medium and may  
5 be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange  
10 chromatography, affinity chromatography, or the like.

Cloning and expression of a transglutaminase enzyme from  
*Phytophthora cactorum*

15

**MATERIALS AND METHODS**

**Deposited organism:** *Escherichia coli* DSM 10256 containing the plasmid comprising the full length DNA sequence,  
20 coding for the transglutaminase of the invention, in the shuttle vector pYES 2.0.

**Yeast strain:** The *Saccharomyces cerevisiae* strain used was W3124 (MAT $\alpha$ ; ura 3-52; leu 2-3, 112; his 3-D200; pep  
25 4-1137; prc1::HIS3; prb1::LEU2; cir+).

**Plasmids:**

The *Aspergillus* expression vector pHD414 is a derivative of the plasmid p775 (described in EP 238 023). The  
30 construction of pHD414 is further described in WO 93/11249.

pYES 2.0 (Invitrogen)

35 **Isolation of the DNA sequence shown in SEQ ID No. 1:**  
The full length DNA sequence, comprising the cDNA sequence shown in SEQ ID No. 1 coding for the transglutaminase of the invention, can be obtained from the deposited organism *Escherichia coli* DSM 10256 by

extraction of plasmid DNA by methods known in the art (Sambrook et al.).

- Extraction of total RNA** was performed with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion, and isolation of poly(A)<sup>+</sup>RNA was carried out by oligo(dT)-cellulose affinity chromatography using the procedures described in WO 94/14953.
- 10 cDNA synthesis:** Double-stranded cDNA was synthesized from 5 µg poly(A)<sup>+</sup> RNA by the RNase H method (Gubler and Hoffman, Sambrook et al.) using the hair-pin modification developed by F. S. Hagen (pers. comm.). The poly(A)<sup>+</sup> RNA (5 µg in 5 µl of DEPC-treated water) was heated at 70°C
- 15** for 8 min. in a pre-siliconized, RNase-free Eppendorph tube, quenched on ice and combined in a final volume of 50 µl with reverse transcriptase buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, Bethesda Research Laboratories) containing 1 mM of dATP, dGTP and
- 20** dTTP and 0.5 mM 5-methyl-dCTP (Pharmacia), 40 units human placental ribonuclease inhibitor (RNasin, Promega), 1.45 µg of oligo(dT)<sub>18</sub>-Not I primer (Pharmacia) and 1000 units SuperScript II RNase H reverse transcriptase (Bethesda Research Laboratories). First-strand cDNA was
- 25** synthesized by incubating the reaction mixture at 45°C for 1 hour. After synthesis, the mRNA:cDNA hybrid mixture was gelfiltrated through a MicroSpin S-400 HR (Pharmacia) spin column according to the manufacturer's instructions.
- 30** After the gelfiltration, the hybrids were diluted in 250 µl second strand buffer (20 mM Tris-Cl, pH 7.4, 90 mM KCl, 4.6 mM MgCl<sub>2</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.16 mM βNAD<sup>+</sup>) containing 200 µM of each dNTP, 60 units *E. coli* DNA polymerase I (Pharmacia), 5.25 units RNase H (Promega)
- 35** and 15 units *E. coli* DNA ligase (Boehringer Mannheim). Second strand cDNA synthesis was performed by incubating the reaction tube at 16°C for 2 hours and additional 15 min. at 25°C. The reaction was stopped by addition of EDTA to a final concentration of 20 mM followed by phenol

and chloroform extractions.

**Mung bean nuclease treatment:** The double-stranded cDNA was precipitated at -20°C for 12 hours by addition of 2  
5 vols 96% EtOH, 0.2 vol 10 M NH<sub>4</sub>Ac, recovered by centrifugation, washed in 70% EtOH, dried and resuspended in 30 µl Mung bean nuclease buffer (30 mM NaAc, pH 4.6, 300 mM NaCl, 1 mM ZnSO<sub>4</sub>, 0.35 mM DTT, 2% glycerol) containing 25 units Mung bean nuclease (Pharmacia). The  
10 single-stranded hair-pin DNA was clipped by incubating the reaction at 30°C for 30 min., followed by addition of 70 µl 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, phenol extraction and precipitation with 2 vols of 96% EtOH and 0.1 vol 3 M NaAc, pH 5.2 on ice for 30 min.

15

**Blunt-ending with T4 DNA polymerase:** The double-stranded cDNAs were recovered by centrifugation and blunt-ended in 30 µl T4 DNA polymerase buffer (20 mM Tris-acetate, pH 7.9, 10 mM MgAc, 50 mM KAc, 1 mM DTT) containing 0.5 mM  
20 of each dNTP and 5 units T4 DNA polymerase (New England Biolabs) by incubating the reaction mixture at 16°C for 1 hour. The reaction was stopped by addition of EDTA to a final concentration of 20 mM, followed by phenol and chloroform extractions, and precipitation for 12 hours at  
25 -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

**Adaptor ligation, Not I digestion and size selection:** After the fill-in reaction the cDNAs were recovered by  
30 centrifugation, washed in 70% EtOH and dried. The cDNA pellet was resuspended in 25 µl ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM ATP) containing 2.5 µg non-palindromic BstXI adaptors (Invitrogen) and 30 units T4 ligase (Promega) and  
35 incubated at 16°C for 12 hours. The reaction was stopped by heating at 65°C for 20 min. and then cooling on ice for 5 min. The adapted cDNA was digested with Not I restriction enzyme by addition of 20 µl water, 5 µl 10x Not I restriction enzyme buffer (New England Biolabs) and

50 units Not I (New England Biolabs), followed by incubation for 2.5 hours at 37°C. The reaction was stopped by heating at 65°C for 10 min. The cDNAs were size-fractionated by gel electrophoresis on a 0.8% SeaPlaque GTG low melting temperature agarose gel (FMC) in 1x TBE to separate unligated adaptors and small cDNAs. The cDNA was size-selected with a cut-off at 0.7 kb and rescued from the gel by use of  $\beta$ -Agarase (New England Biolabs) according to the manufacturer's instructions and precipitated for 12 hours at -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

**Construction of libraries:** The directional, size-selected cDNA was recovered by centrifugation, washed in 70% EtOH, dried and resuspended in 30  $\mu$ l 10 mM Tris-Cl, pH 7.5, 1 mM EDTA. The cDNAs were desalted by gel filtration through a MicroSpin S-300 HR (Pharmacia) spin column according to the manufacturer's instructions. Three test ligations were carried out in 10  $\mu$ l ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM ATP) containing 5  $\mu$ l double-stranded cDNA (reaction tubes #1 and #2), 15 units T4 ligase (Promega) and 30 ng (tube #1), 40 ng (tube #2) and 40 ng (tube #3, the vector background control) of BstXI-NotI cleaved pYES 2.0 vector. The ligation reactions were performed by incubation at 16°C for 12 hours, heating at 70°C for 20 min. and addition of 10  $\mu$ l water to each tube. 1  $\mu$ l of each ligation mixture was electroporated into 40  $\mu$ l electrocompetent *E. coli* DH10B cells (Bethesda research Laboratories) as described (Sambrook et al.). Using the optimal conditions a library was established in *E. coli* consisting of pools containing 15.000-30.000 colony forming units. Each pool of transformed *E. coli* was spread on LB+ampicillin agar plates giving 15.000-30.000 colonies/plate after incubation at 37°C for 24 hours. 20 ml LB+ampicillin was added to the plate and the cells were suspended herein. The cell suspension was shaken in a 50 ml tube for 1 hour at 37°C. Plasmid DNA was isolated from the cells according to the manufacturer's instructions using QIAGEN

plasmid kit and stored at -20°C.

1 µl aliquots of purified plasmid DNA (100 ng/µl) from individual pools were transformed into *S. cerevisiae* W3124 by electroporation (Becker and Guarante) and the transformants were plated on SC agar containing 2% glucose and incubated at 30°C.

**Identification of positive colonies:** After 3-5 days of growth, the agar plates were replica plated onto a set of SC-variant agar plates. These plates were incubated for 6-8 days at 30°C.

Round (diameter 8.2 cm) Immobilon PVDF Transfer Membranes for protein blotting (Millipore) were wetted for 1-3 seconds in 96% EtOH and rinsed in water for 1 min. The membranes were incubated for 2 hours in 2% N,N-dimethylcasein, 150 mM NaCl, 0.1 M Trisbuffer pH 7.5 and washed twice (1 min.) in 150 mM NaCl, 0.1 M Trisbuffer pH 7.5.

A casein saturated membrane was placed on each SC-variant agar plate with yeast colonies. The plate was incubated at 30°C over night with 1 ml 0.5 mM 5-(biotinamido)-pentylamine (Pierce), 0.1 M Trisbuffer pH 7.5, 50 mM CaCl<sub>2</sub>. After 3 washes (15 min.) in 0.1 M Na<sub>3</sub>PO<sub>4</sub>/H<sub>3</sub>PO<sub>4</sub> buffer pH 6.5 the membrane was incubated for 1 hour at room temperature with 10 ml 0.17 µg/ml peroxidase-labeled Streptavidin (Kirkegaard & Perry Laboratories Inc.). After further 3 washes (15 min.) in 0.1 M Na<sub>3</sub>PO<sub>4</sub>/H<sub>3</sub>PO<sub>4</sub> buffer pH 6.5 the membrane was incubated at room temperature with 1 ml 2 mM ABTS (Sigma), 1 mM H<sub>2</sub>O<sub>2</sub>, 0.1 M Na<sub>3</sub>PO<sub>4</sub>/H<sub>3</sub>PO<sub>4</sub> buffer pH 6.5 until transglutaminase positive colonies were identified by a green or lilac zone.

35

Cells from enzyme-positive colonies were spread for single colony isolation on agar, and an enzyme-producing single colony was selected for each of the transglutaminase-producing colonies identified.



Characterization of positive clones: The positive clones were obtained as single colonies, the cDNA inserts were amplified directly from the yeast colony using biotinylated polylinker primers, purified by magnetic beads (Dynabead M-280, Dynal) system and characterized individually by sequencing the 5'-end of each cDNA clone using the chain-termination method (Sanger et al.) and the Sequenase system (United States Biochemical).

- 10 Isolation of a cDNA gene for expression in *Aspergillus*:  
A transglutaminase-producing yeast colony was inoculated into 20 ml YPD broth in a 50 ml glass test tube. The tube was shaken for 2 days at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm.

15

- DNA was isolated according to WO 94/14953 and dissolved in 50 µl water. The DNA was transformed into *E. coli* by standard procedures. Plasmid DNA was isolated from *E. coli* using standard procedures, and analyzed by  
20 restriction enzyme analysis. The cDNA insert was excised using appropriate restriction enzymes and ligated into an *Aspergillus* expression vector.

#### Transformation of *Aspergillus oryzae* or *Aspergillus niger*

25

Protoplasts may be prepared as described in WO 95/02043, p. 16, line 21 - page 17, line 12.

- 100 µl of protoplast suspension is mixed with 5-25 µg of  
30 the appropriate DNA in 10 µl of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM CaCl<sub>2</sub>). Protoplasts are mixed with p3SR2 (an *A. nidulans* amdS gene carrying plasmid). The mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM CaCl<sub>2</sub>,  
35 and 10 mM Tris-HCl, pH 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol.

After one more sedimentation the protoplasts are spread on minimal plates (Cove) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is repeated and spores of a single colony after the second reisolation is stored as a defined transformant.

10 **Test of *A. oryzae* transformants**

Each of the transformants were inoculated in 10 ml YPM and propagated. After 2-5 days of incubation at 37°C, 10 ml supernatant was removed. The transglutaminase activity was identified by the 5-(biotinamido)-pentylamine plate assay described above and the Putrescine assay described in Example 1 below.

**Hybridisation conditions (to be used in evaluating property ii) of the DNA construct of the invention):**

20 Suitable conditions for determining hybridization between a DNA or RNA or an oligonucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (standard saline citrate) for 10 min. and

25 prehybridizing of the filter in a solution of 5 x SSC (Sambrook et al., 1989), 5 x Denhardt's solution (Sambrook et al., 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al., 1989), followed by hybridization in the same solution

30 containing a random-primed (Feinberg and Vogelstein, 1983) <sup>32</sup>P-dCTP labelled (specific activity > 1 x 10<sup>9</sup> cpm/µg) probe for 12 h at ~45°C. The filter is then washed two times for 30 minutes in 2 x SSC, 0.5% SDS at a temperature preferably not higher than 45°C, more

35 preferably not higher than 50°C, even more preferably not higher than 55°C, even more preferably not higher than 60°C, most preferably not higher than 65°C, especially not higher than 70°C, more preferably not higher than 75°C.

A suitable DNA or RNA or an oligonucleotide probe to be used in the hybridization may be prepared on the basis of the DNA sequence shown in SEQ ID No. 1, or on basis of the deduced amino acid sequence shown in SEQ ID No.2.

5

**Immunological cross-reactivity:** Antibodies to be used in determining immunological cross-reactivity may be prepared by use of a purified transglutaminase. More specifically, antiserum against the transglutaminase of the invention may be raised by immunizing rabbits (or other rodents) according to the procedure described by N. Axelsen et al., Chapter 23, or A. Johnstone and R. Thorpe. Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation  $((\text{NH}_4)_2 \text{SO}_4)$ , followed by dialysis and ion exchange chromatography, e.g. on DEAE-Sephadex. Immunochemical characterization of proteins may be done either by Outcherlony double-diffusion analysis (O. Ouchterlony), by crossed immunoelectrophoresis (N. Axelsen et al., Chapters 3 and 4), or by rocket immunoelectrophoresis (N. Axelsen et al., Chapter 2).

#### Media

YPD: 10 g yeast extract, 20 g peptone,  $\text{H}_2\text{O}$  to 900 ml.

25 Autoclaved, 100 ml 20% glucose (sterile filtered) added.

YPM: 10 g yeast extract, 20 g peptone,  $\text{H}_2\text{O}$  to 900 ml.

Autoclaved, 100 ml 20% maltodextrin (sterile filtered) added.

30

10 x Basal salt: 75 g yeast nitrogen base, 113 g succinic acid, 68 g NaOH,  $\text{H}_2\text{O}$  ad 1000 ml, sterile filtered.

SC-URA: 100 ml 10 x Basal salt, 28 ml 20% casamino acids without vitamins, 10 ml 1% tryptophan,  $\text{H}_2\text{O}$  ad 900 ml, autoclaved, 3.6 ml 5% threonine and 100 ml 20% glucose or 20% galactose added.

35

SC-agar: SC-URA, 20 g/l agar added.

SC-variant agar: 20 g agar, 20 ml 10 x Basal salt, H<sub>2</sub>O ad 900 ml, autoclaved, 10 ml 1% tryptophan, 3.6 ml 5% threonine and 100 ml 20% galactose added.

5

### Compositions of the invention

Although the useful transglutaminase preparation or the recombinant transglutaminase may be added as such it is preferred that it is formulated into a suitable composition. The transglutaminase to be used industrially may be in any form suited for the use in question, e.g. in the form of a dry powder or granulate, in particular a non-dusting granulate, a liquid, in particular a stabilized liquid, or a protected enzyme. Granulates may be produced, e.g. as disclosed in US 4,106,991 and US 4,661,452, and may optionally be coated by methods known in the art. Liquid enzyme preparations may, for instance, be stabilized by adding nutritionally acceptable stabilizers such as a sugar, a sugar alcohol or another polyol, lactic acid or another organic acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216. The enzyme preparation of the invention may also comprise a preservative.

25

Normally, for inclusion in flour, baking or baked products, meat products, cheese and other milk products, fish products, cosmetics, various gelled food, it may be advantageous that the enzyme preparation is in the form of a dry product, e.g. a non-dusting granulate, whereas for inclusion together with a liquid it is advantageously in a liquid form.

The recombinant transglutaminase and the transglutaminase preparations of the present invention may also be used in baking for improving the development, elasticity and/or stability of dough and/or the volume, crumb structure and/or anti-staling properties of the baked product. Although the transglutaminase may be used for the

preparation of dough or baked products prepared from any type of flour or meal (e.g. based on rye, barley, oat or maize) the present transglutaminases have been found to be particularly useful in the preparation of dough or

5 baked products made from wheat or comprising substantial amounts of wheat. The baked products produced with a transglutaminase of the invention includes bread, rolls, baguettes and the like. For baking purposes the transglutaminase of the invention may be used as the only

10 or major enzymatic activity, or may be used in combination with other enzymes such as a lipase, an amylase, an oxidase (e.g. glucose oxidase, peroxidase), a laccase and/or a protease.

15 Preferably, the transglutaminase of the invention, especially the recombinant transglutaminase, is used in flour, dough, baked products, meat products, cheese and other milk products, fish products, cosmetics, and various gelled food products in an amount of between 0.01 and

20 100 mg per kg, more preferably of between 0.1 and 50 mg per kg, most preferably between 0.5 and 30 mg per kg, especially between 1 and 10 mg per kg.

Further, it is contemplated that the recombinant

25 transglutaminase and the transglutaminase preparations of the present invention also can exhibit glutaminase activity, i.e. are capable of glutamine-specific deamidation. Accordingly, a protein substrate essentially free of lysine or at least with a very low content of

30 lysine may be subjected to deamidation by applying the transglutaminase of the invention, such as protein being e.g. gluten or a gluten hydrolysate. In another aspect of the invention, the transglutaminases of the invention can be useful for treatment of food products containing

35 gluten, e.g. for improvement of the palability or other properties of bread and other baked food products, or for reducing the allergenicity of food products containing gluten or gluten hydrolysates.

The invention is further illustrated in the following non-limiting examples.

#### EXAMPLE 1

##### 5 Identification of transglutaminase secreting strains belonging to Oomycetes

The oomycetes were inoculated into shake flasks by cutting out 4-8 small pieces of mycelium (5 mm x 5 mm) from  
10 PDA plates (39 g/l potato dextrose agar). The shake flasks contain either SFM-4 (4 g/l meat extract, 4 g/l yeast extract, 40 g/l glucose, 8 g/l tryptone, 0.001 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 tablets/l EBIOS, pH 7.0),  $\frac{1}{2}$ BPX (potato meal 25g/l, barley meal 12.5 g/l, BAN 800 MG 0.013 g/l, Na-  
15 casein 2.5 g/l, soy meal 5 g/l,  $\text{Na}_2\text{HPO}_4$  2.25 g/l, pluronic 0.025 ml/l) or FG-4 (soy meal 30 g/l, maltodextrine 15 g/l, bacto peptone 5 g/l, pluronic 0.2 g/l) medium. The cultures were cultured at 26°C for 5-7 days with shaking. The resulting culture broths were centrifuged 10  
20 minutes at 2300 g to give cell-free culture broths (transglutaminase preparations).

Transglutaminases have been identified in cell-free culture broths of several *Oomycetes* using the assay described in detail below. It was not possible to detect these  
25 transglutaminase activities using the hydroxamate assay (Folk & Cole) as described by others in screening for microbial transglutaminases (EP 0 481504 A1).

30 The assay used is a slightly modified version of the original procedure (Curtis & Lorand). The transglutaminase activity is measured as incorporation of [1,4- $^{14}\text{C}$ ]putrescine into  $\alpha$ -casein. The detection limit of the C14-putrescine incorporation assay was found to be 1/20 of  
35 the detection limit of the hydroxamate assay.

To 20  $\mu\text{l}$  of cell-free culture broth is added 5  $\mu\text{l}$  [1,4- $^{14}\text{C}$ ]putrescine (1.85 MBq/ml in 2% aqueous ethanol; specific activity 4.22 GBq/mmol) and 20  $\mu\text{l}$   $\alpha$ -casein (2% in 50

mM Tris-HCl, 100 mM NaCl, pH 7.5). Incubation takes place for 2 h at room temperature following which 30  $\mu$ l of the assay mixture is spotted onto a small round Whatman 3MM filter. The filter is immediately put into a basket submerged in cold 10% trichloroacetic acid and washed for 20 min to remove excess radioactivity. After this first wash the filters are washed three times with cold 5% trichloroacetic acid, one time with cold ethanol:acetone (50:50, v:v) and one time with cold acetone. Each of these washes takes place for 5 min. In all washing steps the amount of washing liquid should be at least 5 ml/filter. The washed filters are counted directly in scintillation vials.

15 Table 1 shows examples of species belonging to Oomycetes that secrete transglutaminases into the growth medium upon cultivation and the determined enzyme activities are shown in terms of units of transglutaminase activity.

Table 1

No.	Genus	species	Units/ ml	Medium
CBS 701.95	Pythium	irregulare	0.35	SFM-4
CBS 702.95	Pythium	12	2.5	½ BPX
5 CBS 620.94	Pythium	periilum / periplocum	2.5	SFM-4
CBS 703.95	Pythium	intermedium	0.83	SFM-4
CBS 704.95	Pythium	sp.	1.5	½ BPX
	Pythium	torulosum	0.72	½ BPX
CBS 705.95	Pythium	ultimum	0.38	SFM-4
10	Pythium	aphanidermatum	0.37	SFM-4
CBS 618.94	Phytophthora	cactorum	28.3	SFM-4
	Phytophthora	palmivora	5.6	SFM-4
	Phytophthora	cinnamomi	4.9	SFM-4
15 CBS 651.94	Phytophthora	cryptogea	10.0	FG-4

Units: An enzyme activity which incorporates 1 nmol [<sup>14</sup>C]-putrescine per hour is defined as 1 U.

## 20 EXAMPLE 2

### Casein polymerisation

The ability of the transglutaminase present in  
 25 *Phytophthora cactorum* culture broth to polymerize α-casein was investigated using SDS polyacrylamide gel electrophoresis (SDS-PAGE).

To 20 μl of *Phytophthora cactorum* culture broth was added  
 30 20 μl 1.5% α-casein in 0.2 M Tris-HCl, pH 7.5. The mixture was incubated for 2 h at room temperature.



Control samples where the culture broth or the  $\alpha$ -casein were substituted with water were incubated in parallel.

5 SDS-PAGE of 10  $\mu$ l of each of the three samples clearly showed that only the *Phytophthora cactorum* culture broth converted the  $\alpha$ -casein to high molecular weight polymers.

### EXAMPLE 3

10

**Activity dependence in the presence of cysteine or  $\text{Ca}^{2+}$ -ions at different temperatures**

15 The effect of reducing agents such as cysteine and  $\text{Ca}^{2+}$ -ions on the transglutaminase activity at different temperatures was investigated using a modification of the putrescine assay described in example 1.

20 The transglutaminase preparations were concentrated approximately 10 times using a Macrosep<sup>TM</sup> concentrator from Filtron. Following the samples were diluted 10 times in either:

- a) 50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.5;
- b) 50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 1 mM
- 25 cysteine, pH 7.5;
- c) 50 mM Tris-HCl, 100 mM NaCl, 5 mM  $\text{CaCl}_2$ , pH 7.5; or
- d) 50 mM Tris-HCl, 100 mM NaCl, 1 mM cysteine, 5 mM  $\text{CaCl}_2$ , pH 7.5.

30 For activity determination incubation took place for 1 hour at room temperature, 40°C and 55°C, respectively.

35 The tables below show the activity dependencies of the different parameters. The enzyme activities are given in relative activities. The activity obtained in buffer + EDTA at room temperature is set to 100. The activity of transglutaminase is dependent on calcium and in most cases the activity measured in the culture broth is further increased by the presence of cysteine.

Strain: *Phytophthora cactorum*, CBS 618.94

5		50 mM Tris-HCl, 100 mM NaCl, pH 7.5			
	Temperature	2mM EDTA	2mM EDTA +1mM Cys	+5mM Ca <sup>2+</sup>	+ 1 mM Cys + 5 mM Ca <sup>2+</sup>
	Room temp.	100	125	986	991
	40°C	68	85	1954	2350
	55°C	70	58	1073	829

Strain: *Phytophthora cryptogea*, CBS 651.94

15		50 mM Tris-HCl, 100 mM NaCl, pH 7.5			
	Temperature	2mM EDTA	2mM EDTA +1mM Cys	+ 5 mM Ca <sup>2+</sup>	+ 1 mM Cys + 5 mM Ca <sup>2+</sup>
	Room temp.	100	115	1267	2527
	40°C	69	69	4372	7423
	55°C	78	143	3865	5518

Strain: *Pythium* sp., CBS 702.95

5		50 mM Tris-HCl, 100 mM NaCl, pH 7.5			
	Temperature	2mM EDTA	2mM EDTA +1mM Cys	+ 5 mM $\text{Ca}^{2+}$	+ 1 mM Cys + 5 mM $\text{Ca}^{2+}$
	Room temp.	100	57	487	991
	40°C	0	0	3216	5773
	55°C	100	96	4191	5896

10

Strain: *Pythium irregulare*, CBS 701.95

15		50 mM Tris-HCl, 100 mM NaCl, pH 7.5			
	Temperature	2mM EDTA	2mM EDTA +1mM Cys	+ 5 mM $\text{Ca}^{2+}$	+ 1 mM Cys + 5 mM $\text{Ca}^{2+}$
	Room temp.	100	110	87	86
	40°C	167	168	462	450
	55°C	50	43	130	114

20

Strain: *Pythium ultimum*, CBS 705.95

50 mM Tris-HCl, 100 mM NaCl, pH 7.5				
Temperature	2mM EDTA	2mM EDTA +1mM Cys	+ 5 mM Ca <sup>2+</sup>	+ 1 mM Cys + 5 mM Ca <sup>2+</sup>
Room temp.	100	93	107	141
40°C	142	164	416	483
55°C	15	22	89	88

10

Strain: *Pythium intermedium*, CBS 703.95

50 mM Tris-HCl, 100 mM NaCl, pH 7.5				
Temperature	2mM EDTA	2mM EDTA +1mM Cys	+ 5 mM Ca <sup>2+</sup>	+ 1 mM Cys + 5 mM Ca <sup>2+</sup>
Room temp.	100	138	459	2438
40°C	129	142	3872	6117
55°C	181	180	733	1716

15

20

#### 25 Example 4

#### pH Dependency of Oomycetes transglutaminases

The pH dependency of the transglutaminase activity

Strain: *Pythium ultimum*, CBS 705.95

50 mM Tris-HCl, 100 mM NaCl, pH 7.5				
Temperature	2mM EDTA	2mM EDTA +1mM Cys	+ 5 mM Ca <sup>2+</sup>	+ 1 mM Cys + 5 mM Ca <sup>2+</sup>
Room temp.	100	93	107	141
40°C	142	164	416	483
55°C	15	22	89	88

10

Strain: *Pythium intermedium*, CBS 703.95

50 mM Tris-HCl, 100 mM NaCl, pH 7.5				
Temperature	2mM EDTA	2mM EDTA +1mM Cys	+ 5 mM Ca <sup>2+</sup>	+ 1 mM Cys + 5 mM Ca <sup>2+</sup>
Room temp.	100	138	459	2438
40°C	129	142	3872	6117
55°C	181	180	733	1716

15

20

25 Example 4

#### pH Dependency of Oomycetes transglutaminases

The pH dependency of the transglutaminase activity

present in the transglutaminase preparation of *Pythium irregulare* (CBS 701.95), *Pythium sp.* (CBS 702.95), *Pythium periillum* (or *P. periplocum*) (CBS 620.94), *Pythium intermedium* (CBS 703.95), *Pythium sp.* (CBS 704.95), *Pythium ultimum* (CBS 705.95), *Phytophthora cactorum* (CBS 618.94/IFO 30474) and *Phytophthora cryptogea* (CBS 651.94) was investigated using a modification of the putrescine assay described in example 1.

- 10 A 4%  $\alpha$ -casein solution was made in 50 mM Tris-HCl, 100 mM NaCl, 5 mM  $\text{CaCl}_2$ , 1 mM cysteine, pH 7.5 and diluted 1:1 in a modified 200 mM Britton-Robinson buffer (0.1M  $\text{CH}_3\text{COOH}$ , 0.2 M  $\text{H}_3\text{BO}_3$ ) at the pH values mentioned below.
- 15 For pH dependency determination incubation takes place at room temperature for 1 hour at pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 or 9.0, respectively.

The table below shows the pH dependencies of the

- 20 Oomycetes transglutaminases. The stated enzyme activities are relative activities.

pH							
Strains	6.0	6.5	7.0	7.5	8.0	8.5	9.0
5 <i>Pythium irregulare</i> , CBS 701.95	20	24	36	46	62	100	46
<i>Pythium</i> <i>sp.</i> , CBS 702.95	9	16	27	31	48	93	100
10 <i>Pythium intermedium</i> , CBS 703.95	63	90	99	100	95	54	25
15 <i>Pythium</i> <i>sp.</i> , CBS 704.95	23	33	41	72	95	100	78
<i>Pythium ultimum</i> , CBS 705.95	28	62	68	68	100	93	69
20 <i>Phytophthora cactorum</i> , CBS 618.94	28	38	46	59	74	100	92
25 <i>Phytophthora cryptogea</i> , CBS 651.94	63	78	86	100	99	93	56

## 30 EXAMPLE 5

Cloning and expression of a transglutaminase from  
*Phytophthora cactorum*, CBS 618.94 and IFO 30474

mRNA was isolated from *Phytophthora cactorum*, CBS 618.94

and IFO 30474, grown in SFM-4 fermentation medium with agitation to ensure sufficient aeration. Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80°C. A library from *P. cactorum*, CBS 618.94 or IFO 30474 consisting of approx. 5 9x10<sup>5</sup> individual clones was constructed in *E. coli* as described with a vector background of 1%. Plasmid DNA from some of the pools was transformed into yeast, and 50-100 plates containing 250-400 yeast colonies were 10 obtained from each pool.

Transglutaminase-positive colonies were identified and isolated on agar plates with the 5-(biotinamido)-pentylamine assay. cDNA inserts were amplified directly 15 from the yeast colonies and characterized as described in the Materials and Methods section above. The DNA sequence of the cDNA encoding the transglutaminase is shown in SEQ ID No. 1 and the corresponding amino acid sequence is shown in SEQ ID No. 2.

20

The cDNA is obtainable from the plasmid in DSM 10256.

Total DNA was isolated from a yeast colony and plasmid DNA was rescued by transformation of *E. coli* as described 25 above. In order to express the transglutaminase in *Aspergillus*, the DNA was digested with HindIII/XbaI, size fractionated on gel, and a fragment corresponding to the transglutaminase gene was purified. The gene was subsequently ligated to HindIII/XbaI digested pHD414 30 resulting in the plasmid pA2TG3.

After amplification of the DNA in *E. coli* the plasmid was transformed into *Aspergillus oryzae* as described above.

### 35 Test of *A. oryzae* transformants

Each of the transformants were tested for enzyme activity as described above. Some of the transformants had transglutaminase activity which was significantly larger than the *Aspergillus oryzae* background. This demonstrates



efficient expression of the transglutaminase in *Aspergillus oryzae*.

#### **Fed batch fermentation**

- 5 Fermentations were carried out as fed-batch processes with maltose sirup as carbon source and ammonia as nitrogen source. The batch phase was carried out at pH 6.5 and the pH was increased to 7.5 during the fed-batch phase. The temperature was maintained at 34°C during the  
10 entire process.

#### **EXAMPLE 6**

- Production of the transglutaminase from *Phytophthora cactorum*, CBS 918.94/IFO 60474**  
15

- Phytophthora cactorum*, CBS 618.94/IFO 30474, was inoculated into 8 l SFM-4 medium and cultured with shaking at 26 °C for 7 days. The resulting culture broth was  
20 filtered through Miracloth to give 5 l of culture filtrate. The transglutaminase activity in the culture filtrate was 22 units/ml.

#### **25 EXAMPLE 7**

**Purification and characterization of native and recombinant *Phytophthora cactorum* transglutaminase.**

**Transglutaminase activity measured with putrescine assay:**

- 30 The putrescine assay was in principle performed according to Lorand et al.

- The reaction mixture contained: 2  $\mu$ moles of  $\text{CaCl}_2$ , 1  $\mu$ moles of cysteine, 75 nmoles of [ $^{14}\text{C}$ ]-putrescine (4.03 GBq/mmol; Amersham), 0.7 mg of  $\alpha$ -casein, and 0.6  $\mu$ g of  
35 transglutaminase made up to 1 ml with 0.1 M Tris-HCl, pH 7.9. The incubations were performed at ambient temperature. Aliquots of 30  $\mu$ l were withdrawn after 60 min of incubation and spotted onto Whatman 3 MM filters

(D = 2 cm). The filters were immediately put into a basket submerged in ice-cold 10% TCA and washed for 20 min. Following the first wash the filters were washed three times with ice-cold 5% TCA and two times with ice-cold acetone. In each washing step there should be at least 5 ml of washing solution per filter. The filters were dried, put into counting vials containing 8 ml of scintillation fluid (Optiphase, Wallac) and the radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. Each determination was performed in triplicate.

**Partially purification of native *P. cactorum* transglutaminase.**

The culture broth was germ filtrated and concentrated 5 times by ultrafiltration using a Filtron Minisette membrane with 10 kDa cut off. After dialysis against 20 mM Tris-HCl, pH 8.0 the sample was passed through a Q-Sepharose column equilibrated with 20 mM Tris-HCl, pH 8.0. The transglutaminase was eluted from the column using a linear gradient from 0 to 0.5 M sodium chloride. Fractions with transglutaminase activity (putrescine assay) were pooled and concentrated in an Amicon cell equipped with a 10 kDa Diaflo membrane. This preparation of native transglutaminase was only partially pure.

**Purification, specific activity and N-terminal sequencing of recombinant *P. cactorum* transglutaminase.**

The *Aspergillus oryzae* culture broth was germ filtrated and concentrated 5 times by ultrafiltration using a Filtron Minisette membrane with 10 kDa cut off. After dialysis against 50 mM sodium borate, pH 8.0 the sample was passed through a Q-Sepharose column equilibrated with 50 mM sodium borate, pH 8.0. The transglutaminase was eluted from the column using a linear gradient from 0 to 0.5 M sodium chloride. Fractions that gelate casein were pooled and concentrated in an Amicon cell equipped with a

10<sup>3</sup> kDa-Diaflo membrane.

In *Aspergillus oryzae* the recombinant transglutaminase is produced as two forms and from SDS-PAGE the molecular weights are judged to be 57 kDa and 43 kDa, respectively. The ratio between the two forms is dependent on the fermentation time. Early in the fermentation the 57 kDa form dominates but this form is during the fermentation processed to the low molecular weight form. Both forms of the transglutaminase are catalytic active. The specific activity of the recombinant transglutaminase was determined in the putrescine assay and found to be 3,000 U/mg.

N-terminal amino acid sequencing of the two forms of the transglutaminase revealed that the 57 kDa form has a blocked N-terminal and that the 43 kDa form starts at Leu168, cf. SEQ ID No.2.

20

The influence of calcium and cysteine on the activity of recombinant *P. cactorum* transglutaminase.

The effect of calcium and cysteine (used as a reducing agent) was investigated in the putrescine assay. The results presented below are given as relative activities. The activity obtained in buffer at 25°C is set to 100.

The activity of the transglutaminase is dependent on calcium and the activity is not further increased by the presence of cysteine as reducing agent.

5

Temp. (°C)	Buffer	2 mM EDTA	1 mM Cys	2 mM Ca <sup>2+</sup>	1 mM Cys 2 mM Ca <sup>2+</sup>
25	100	15	180	270	280
30	105	10	210	430	490
40	30	10	75	750	780
55	10	5	75	350	350

10 The influence of calcium and cysteine on the gelation of casein by *P. cactorum* transglutaminase.

The influence of calcium and cysteine on the gelation of casein was investigated as described below.

15 The gelation mixture contained 80 mg Hammarsten casein, 2  $\mu$ moles of calcium, 1  $\mu$ mole of cysteine, and approximately 0.03 mg transglutaminase made up to 1 ml with 0.2 M Tris-HCl, pH 7.5. Following incubation overnight at 37 °C the samples were tempered to ambient temperature and the  
20 gelation was judged by visual inspection.

Both native and recombinant transglutaminase are able to gelate casein. Contrary to the native enzyme it is not essential for the recombinant enzyme that cysteine is  
25 present as a reducing agent.

	Buffer	1 mM Cys	2 mM Ca <sup>2+</sup>	1 mM Cys+ 2 mM Ca <sup>2+</sup>
	-*	-	+**	+
5	-	-	-	+

\* - designates no visible gelation.

10 \*\* + designates the formation of a stable gel

# partially purified

#### Temperature profile of *P. cactorum* transglutaminase.

15

The temperature profile was determined using the putrescine assay with 0.1 M sodium borate/acetate buffer, pH 7.9 instead of 0.1 M Tris-HCl, pH 7.9.

20 As can be seen from the table the temperature optimum for both the native and the recombinant transglutaminase is 45 °C.

5       10	Temp. (°C)	Native <i>P. cactorum</i> <sup>*</sup> Relative activity (%)	Recomb. <i>P. cactorum</i> Relative activity (%)
	25	20	30
	30	40	50
	35	60	60
	40	85	75
	45	100	100
	50	75	85
	55	15	25

<sup>\*</sup> partially purified.

**pH profile of recombinant *P. cactorum* transglutaminase.**

15

The pH profile was determined using the putrescine assay with 0.1 M sodium borate/acetate buffer.

**pH optimum of the recombinant *Phytophthora cactorum* transglutaminase is found to be at pH 8.5.**

20

	pH	Recombinant <i>P. cactorum</i> Relative activity (%)
	6.5	10
	7.0	15
	7.5	35
5	8.0	45
	8.5	100
	9.0	85
	9.5	80

10 \* visible precipitate in the substrate

#### EXAMPLE 8

15 Crosslinking of Na-caseinate in solution measured by the  
viscosity increase as function of time

A 9% protein solution was prepared from Na-caseinate (Miprodan 30, MD Foods, Denmark, 87.8% protein). Calcium chloride was dissolved in the solution to a concentration  
20 of 5 mM and pH was adjusted to 7.0, using NaOH. The solution was heated to 40°C.

A Haake Viscosimeter, VT 501 (Haake Mess-Technik GmbH, Germany) was prepared for viscosity measurements at 40°C  
25 by sensor system MV1 at speed range H, speed 3.

To the protein solution was added recombinant *Phytophthora cactorum* transglutaminase, cf. example 7, purified to electrophoretic purity, at a dosage of 0.08%  
30 (weight of enzyme/weight of protein). The solution was immediately transferred to the viscosimeter for measurement. The viscosity of a control solution without enzyme

addition was subsequently measured.

Results: Viscosity (mPa\*s) as function of time:

5	Time (minutes)	Caseinate solution + enzyme	Control
	2.66	17.5	21
	7.33	25.4	22.8
	12	36.9	27.2
	24	88.6	35
10	36	186.9	41.1
	48	355.3	47.3
	60	800.2	50.7

The casein solution with enzyme solidified subsequently to a gel within a few minutes, while the viscosity of the control remained constant at 53 mPa\*s for 120 minutes.

#### EXAMPLE 9

#### 20 Transglutaminase for Gluten Strengthening

The strengthening effect of a given dough conditioner on wheat flour dough or gluten dough may be measured by dynamic rheological measurements. These measurements are able to show the strength of a dough, under oscillation. Both wheat flour dough and gluten dough are viscoelastic materials. In oscillatory measurements, the viscoelastic properties of a wheat dough and a gluten dough can be divided into two components, the dynamic shear storage modulus  $G'$  and the dynamic shear loss modulus  $G''$ . The ratio of the loss and the storage moduli is numerically equal to the tangent of the viscoelastic phase angle  $\delta$ . An increase in the storage modulus  $G'$  and a decrease in



the phase angle  $\delta$  indicate a stronger and more elastic dough.

The dynamic shear storage modulus  $G'$  and the viscoelastic phase angle  $\delta$  were measured in the gluten from 3 doughs, which were treated with the recombinant transglutaminase described in example 6 in two dosages, i.e. 4 mg and 10 mg, respectively. The transglutaminase was added to the flour before dough mixing. The gluten was washed out of the flour dough containing the conditioner after the flour dough had been incubated at 32°C for 1½ hours. The results of the tests are shown in the table below where the measured values of  $G'$  and  $\delta$  resulting from the inclusion of 4 mg and 10 mg enzyme per kg of flour, respectively, are presented as index values relative to the control dough (index 100) with no transglutaminase inclusion.

Dosage of transglutaminase	$G'$ Index	$\delta$ Index
4 mg	135	117
10 mg	167	131

From the results it is surprisingly seen that the storage modulus,  $G'$ , is significantly higher when transglutaminase is present in the dough compared to control without the enzyme. This indicates that the gluten, and thereby also the dough, is significantly strengthened by the action of the enzyme.

Further, it is shown that the viscoelastic phase angle,  $\delta$ , is lowered relative to the control when transglutaminase is present in the dough, indicating that a more elastic rheological property of the gluten and thereby the dough is achieved by the action of the enzyme.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

- (A) NAME: Novo Nordisk A/S
- (B) STREET: Novo Alle
- (C) CITY: DK-2880 Bagsvaerd
- (E) COUNTRY: Denmark
- (F) POSTAL CODE (ZIP): DK-2880
- (G) TELEPHONE: +45 44 44 88 88
- (H) TELEFAX: +45 44 49 32 56
- (I) TELEX: 37304

## (ii) TITLE OF INVENTION: TITLE

## (iii) NUMBER OF SEQUENCES: 2

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1901 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Phytophthora cactorum
- (B) STRAIN: CBS 618.94

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 46..1765

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CGACATCGAG AAGATTACAA ACTCATTGTT GCAGGTTTCA CAACC ATG GTC TAC   54
                               Met Val Tyr
                               1

TCA CCC AGC TCC TAC CTC ATC TCC GCC GCC GTG GCT GCG GTG GCC TTC  102
Ser Pro Ser Ser Tyr Leu Ile Ser Ala Ala Val Ala Ala Val Ala Phe
      5                10                15

CAG ATT CAG CAA GCG ACT GCC GGA TCG CTG TAC TAC GGG GCG TTC TCC  150
Gln Ile Gln Gln Ala Thr Ala Gly Ser Leu Tyr Tyr Gly Ala Phe Ser
    20                25                30                35

GTG TCC GAC ACG GAT GGC AAA ATC AGC AAC GAC TCT CCT CTT GTC GGT  198
Val Ser Asp Thr Asp Gly Lys Ile Ser Asn Asp Ser Pro Leu Val Gly
                40                45                50

ACC GAA ATT TCC GAC CAG GAC TGC GCC ATC GAG GTG GAG GTC GAC CCG  246
Thr Glu Ile Ser Asp Gln Asp Cys Ala Ile Glu Val Glu Val Asp Pro
                55                60                65

ACG CTG CCG GAC ATC ACG ACC ATC TCG ACG GTG CCG GTG ACC TAC CCT  294
Thr Leu Pro Asp Ile Thr Thr Ile Ser Thr Val Pro Val Thr Tyr Pro
                70                75                80

GAC CTG CTG GCC AAC TTG ACG ACG GCT CCG TCG GAG CCG GTG TTC TCA  342
Asp Leu Leu Ala Asn Leu Thr Thr Ala Pro Ser Glu Pro Val Phe Ser
    85                90                95

AAG GTG GGC ACG GTG ATC ATG TCG GAG GAG ACC CCC GCC ACC GAC GCC  390
Lys Val Gly Thr Val Ile Met Ser Glu Glu Thr Pro Ala Thr Asp Ala
   100                105                110                115

GAC CAG GAC GCG TAC ATC GAC TCG ACG CTT CCG TGG ATT GGC ACT GGT  438
Asp Gln Asp Ala Tyr Ile Asp Ser Thr Leu Pro Trp Ile Gly Thr Gly
                120                125                130

ACG CCG ACC AAG ACG GGT GTG GAG AAG ACC GCC AAG GAC TGC GCT ACT  486
Thr Pro Thr Lys Thr Gly Val Glu Lys Thr Ala Lys Asp Cys Ala Thr
                135                140                145

GGG TGG GAG GAG ACC GCG GCC GGC GAT AAG CTC CAG GAG AAG CTC GAA  534
Gly Trp Glu Glu Thr Ala Ala Gly Asp Lys Leu Gln Glu Lys Leu Glu
    150                155                160

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AAG AAG CGT CGC CTG GAG GAA AAC ACG AAC AGG GAT ATC GCT AGG CTC 582  
 Lys Lys Arg Arg Leu Glu Glu Asn Thr Asn Arg Asp Ile Ala Arg Leu  
 165 170 175

GAG GCC TAC TTT GGC ACC AAG ATG GAG ATG ACC CTG AAG GAC CTG CCT 630  
 Glu Ala Tyr Phe Gly Thr Lys Met Glu Met Thr Leu Lys Asp Leu Pro  
 180 185 190 195

ACC CAG GGT GTC CAC ACA CCG TCG CCG TGG GCT GGA CCG TAC TGG CCG 678  
 Thr Gln Gly Val His Thr Pro Ser Pro Trp Ala Gly Pro Tyr Trp Pro  
 200 205 210

ACT TAC CAG GAC AGT ATC AAC GTT GTC TGG AGC GAG GGA GAA GCC AGC 726  
 Thr Tyr Gln Asp Ser Ile Asn Val Val Trp Ser Glu Gly Glu Ala Ser  
 215 220 225

CCC GCT GAG AAG TAC GCC AAG GCT TTC GGT CTG GAC GTG ACG GAC TTC 774  
 Pro Ala Glu Lys Tyr Ala Lys Ala Phe Gly Leu Asp Val Thr Asp Phe  
 230 235 240

ATG GAC AAG GTG TCG AAG GAC AAT GGT GTG GAC TCT CAG AGC AAA CGC 822  
 Met Asp Lys Val Ser Lys Asp Asn Gly Val Asp Ser Gln Ser Lys Arg  
 245 250 255

AGA CAG TGC CAG ACT GAC GAG GGA TGC GAG TCC CTT AAC AAC GCC AGC 870  
 Arg Gln Cys Gln Thr Asp Glu Gly Cys Glu Ser Leu Asn Asn Ala Ser  
 260 265 270 275

AAG TGC GCC ATT CGT GCC GGC AAG ACC TCG GGC TAC TGC ATC CCG ACG 918  
 Lys Cys Ala Ile Arg Ala Gly Lys Thr Ser Gly Tyr Cys Ile Pro Thr  
 280 285 290

TGG TTC GGG ATC TGC CAC GCT TGG GCC CCG GCT GCC ATT CTC GAG GCA 966  
 Trp Phe Gly Ile Cys His Ala Trp Ala Pro Ala Ala Ile Leu Glu Ala  
 295 300 305

GAG CCG ACC TGC CCG GTG ACG CAC AAC GGC GTG ACG TTC CAG CCG ATT 1014  
 Glu Pro Thr Cys Pro Val Thr His Asn Gly Val Thr Phe Gln Pro Ile  
 310 315 320

GAC ATC AAG GGG CTG ATC TCG GAC GTC TAC GAT GGC GCA GGT GTG GCA 1062  
 Asp Ile Lys Gly Leu Ile Ser Asp Val Tyr Asp Gly Ala Gly Val Ala  
 325 330 335

ACG GTT TTC ACG GGT GCC CGG TAC AAC GGC GGT GAC GAT GCT GCC GAT 1110  
 Thr Val Phe Thr Gly Ala Arg Tyr Asn Gly Gly Asp Asp Ala Ala Asp  
 340 345 350 355

GAG TAT GGC CGT CAC ACG AAT GCC GCC TAC CGC GAC CTG AAC CCT GCC 1158  
 Glu Tyr Gly Arg His Thr Asn Ala Ala Tyr Arg Asp Leu Asn Pro Ala  
 360 365 370

TAC TTC CAC ATT GCG TCT GCC AAT ATC CTG GGC AAG CTA AAC GCT ACA 1206  
 Tyr Phe His Ile Ala Ser Ala Asn Ile Leu Gly Lys Leu Asn Ala Thr  
 375 380 385

TTT GTT GCT GAC GTC GAC GCC GCC GCA GAA GTG TGG AAC CAG CCC GTG 1254  
 Phe Val Ala Asp Val Asp Ala Ala Ala Glu Val Trp Asn Gln Pro Val  
 390 395 400

CGC GGT TTC AAG GTG TTC GAG CAG ACC GCC ATG TCG CTC GAG GAG GCC 1302  
 Arg Gly Phe Lys Val Phe Glu Gln Thr Ala Met Ser Leu Glu Glu Ala  
 405 410 415

GCT CAG ACC TTC TAC GGC CTT GAG GAG TAC CCG TGG AAT GCC GCC GCC 1350  
 Ala Gln Thr Phe Tyr Gly Leu Glu Glu Tyr Pro Trp Asn Ala Ala Ala  
 420 425 430 435

AAG AGC ATT GTG TAC GTC AAG TCG CGT CTC TCG TGG ATC TTC GAG ACG 1398  
 Lys Ser Ile Val Tyr Val Lys Ser Arg Leu Ser Trp Ile Phe Glu Thr  
 440 445 450

TAC ACC GAC GGT GGC CTG GTG GCC TCG GGT GAG ATC AAC CGA TAC ACA 1446  
 Tyr Thr Asp Gly Gly Leu Val Ala Ser Gly Glu Ile Asn Arg Tyr Thr  
 455 460 465

ACG GGC AAG TAC TAC TAC TAC CTT CTG GAG CTG GAC GAT GCT GGT GAG 1494  
 Thr Gly Lys Tyr Tyr Tyr Tyr Leu Leu Glu Leu Asp Asp Ala Gly Glu  
 470 475 480

ATC ATT GGC GGT GAG TGG GTT TAC GAT TCG GAC AGC GAC CAC CCT GAC 1542  
 Ile Ile Gly Gly Glu Trp Val Tyr Asp Ser Asp Ser Asp His Pro Asp  
 485 490 495

TTC CTG TGG GTG CCC AAG GCG AAG CCT GCT GCG GAC ACG GTG ACC AGC 1590  
 Phe Leu Trp Val Pro Lys Ala Lys Pro Ala Ala Asp Thr Val Thr Ser  
 500 505 510 515

ATT GGC CTG AGC TAC GCG GAC GTG AGC ATG CTT CTG GAG AAA TCC GTC 1638  
 Ile Gly Leu Ser Tyr Ala Asp Val Ser Met Leu Leu Glu Lys Ser Val  
 520 525 530

GCT TGC TCC GAC TCC ACT TCG GCT GCC GGC TCC GTG TCG TCC GGA TCG 1686  
 Ala Cys Ser Asp Ser Thr Ser Ala Ala Gly Ser Val Ser Ser Gly Ser  
 535 540 545

GTG GGT GAG TCC ACG GAG GCG CCT ACG GAA GTG CCC ACG ACG TCG ACG 1734  
 Val Gly Glu Ser Thr Glu Ala Pro Thr Glu Val Pro Thr Thr Ser Thr  
 550 555 560

AGT GCT CCC ACT TCT GGC AGT GGC GCG CTG T AAGTATGTCG CAGCTCGTCT 1785  
 Ser Ala Pro Thr Ser Gly Ser Gly Ala Leu  
 565 570

TCCGTATTTG TCGTTGCACA TGAATGTGTA GCTTCGTTTA GATTGCCAGC 1835  
 TATTCACAAG AAATGGTTCA AAACATGCAC TAAGTTCAGG TTGTAAAAA 1885  
 AAAAAAAAAA AAAAAA 1901

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 573 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Val Tyr Ser Pro Ser Ser Tyr Leu Ile Ser Ala Ala Val Ala Ala  
 1 5 10 15

Val Ala Phe Gln Ile Gln Gln Ala Thr Ala Gly Ser Leu Tyr Tyr Gly  
 20 25 30

Ala Phe Ser Val Ser Asp Thr Asp Gly Lys Ile Ser Asn Asp Ser Pro  
 35 40 45

Leu Val Gly Thr Glu Ile Ser Asp Gln Asp Cys Ala Ile Glu Val Glu  
 50 55 60

Val Asp Pro Thr Leu Pro Asp Ile Thr Thr Ile Ser Thr Val Pro Val  
 65 70 75 80

Thr Tyr Pro Asp Leu Leu Ala Asn Leu Thr Thr Ala Pro Ser Glu Pro  
 85 90 95



Val Phe Ser Lys Val Gly Thr Val Ile Met Ser Glu Glu Thr Pro Ala  
 100 105 110

Thr Asp Ala Asp Gln Asp Ala Tyr Ile Asp Ser Thr Leu Pro Trp Ile  
 115 120 125

Gly Thr Gly Thr Pro Thr Lys Thr Gly Val Glu Lys Thr Ala Lys Asp  
 130 135 140

Cys Ala Thr Gly Trp Glu Glu Thr Ala Ala Gly Asp Lys Leu Gln Glu  
 145 150 155 160

Lys Leu Glu Lys Lys Arg Arg Leu Glu Glu Asn Thr Asn Arg Asp Ile  
 165 170 175

Ala Arg Leu Glu Ala Tyr Phe Gly Thr Lys Met Glu Met Thr Leu Lys  
 180 185 190

Asp Leu Pro Thr Gln Gly Val His Thr Pro Ser Pro Trp Ala Gly Pro  
 195 200 205

Tyr Trp Pro Thr Tyr Gln Asp Ser Ile Asn Val Val Trp Ser Glu Gly  
 210 215 220

Glu Ala Ser Pro Ala Glu Lys Tyr Ala Lys Ala Phe Gly Leu Asp Val  
 225 230 235 240

Thr Asp Phe Met Asp Lys Val Ser Lys Asp Asn Gly Val Asp Ser Gln  
 245 250 255

Ser Lys Arg Arg Gln Cys Gln Thr Asp Glu Gly Cys Glu Ser Leu Asn  
 260 265 270

Asn Ala Ser Lys Cys Ala Ile Arg Ala Gly Lys Thr Ser Gly Tyr Cys  
 275 280 285

Ile Pro Thr Trp Phe Gly Ile Cys His Ala Trp Ala Pro Ala Ala Ile  
 290 295 300

Leu Glu Ala Glu Pro Thr Cys Pro Val Thr His Asn Gly Val Thr Phe  
 305 310 315 320

Gln Pro Ile Asp Ile Lys Gly Leu Ile Ser Asp Val Tyr Asp Gly Ala  
 325 330 335

Gly Val Ala Thr Val Phe Thr Gly Ala Arg Tyr Asn Gly Gly Asp Asp  
340 345 350

Ala Ala Asp Glu Tyr Gly Arg His Thr Asn Ala Ala Tyr Arg Asp Leu  
355 360 365

Asn Pro Ala Tyr Phe His Ile Ala Ser Ala Asn Ile Leu Gly Lys Leu  
370 375 380

Asn Ala Thr Phe Val Ala Asp Val Asp Ala Ala Ala Glu Val Trp Asn  
385 390 395 400

Gln Pro Val Arg Gly Phe Lys Val Phe Glu Gln Thr Ala Met Ser Leu  
405 410 415

Glu Glu Ala Ala Gln Thr Phe Tyr Gly Leu Glu Glu Tyr Pro Trp Asn  
420 425 430

Ala Ala Ala Lys Ser Ile Val Tyr Val Lys Ser Arg Leu Ser Trp Ile  
435 440 445

Phe Glu Thr Tyr Thr Asp Gly Gly Leu Val Ala Ser Gly Glu Ile Asn  
450 455 460

Arg Tyr Thr Thr Gly Lys Tyr Tyr Tyr Tyr Leu Leu Glu Leu Asp Asp  
465 470 475 480

Ala Gly Glu Ile Ile Gly Gly Glu Trp Val Tyr Asp Ser Asp Ser Asp  
485 490 495

His Pro Asp Phe Leu Trp Val Pro Lys Ala Lys Pro Ala Ala Asp Thr  
500 505 510

Val Thr Ser Ile Gly Leu Ser Tyr Ala Asp Val Ser Met Leu Leu Glu  
515 520 525

Lys Ser Val Ala Cys Ser Asp Ser Thr Ser Ala Ala Gly Ser Val Ser  
530 535 540

Ser Gly Ser Val Gly Glu Ser Thr Glu Ala Pro Thr Glu Val Pro Thr  
545 550 555 560

Thr Ser Thr Ser Ala Pro Thr Ser Gly Ser Gly Ala Leu  
565 570

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

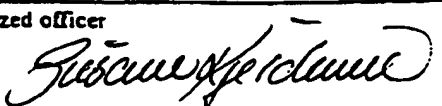
<b>A.</b> The indications made below relate to the microorganism referred to in the description on page <u>4</u> , line <u>17-18</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution <b>DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL-KULTUREN GmbH</b>	
Address of depositary institution (including postal code and country)  <p style="text-align: center;">Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany</p>	
Date of deposit 18 September 1995	Accession Number DSM 10256
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<p>In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).</p>	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

<b>A. The indications made below relate to the microorganism referred to in the description</b> on page <u>3</u> , line <u>30-31</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution <p style="text-align: center;"><b>CENTRAALBUREAU VOOR SCHIMMELCULTURES</b></p>	
Address of depositary institution (including postal code and country) <p style="text-align: center;"><b>Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, the Netherlands</b></p>	
Date of deposit <p style="text-align: center;"><b>19 October 1995</b></p>	Accession Number <p style="text-align: center;"><b>CBS 705.95</b></p>
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<p><b>In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).</b></p>	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

<b>A.</b> The indications made below relate to the microorganism referred to in the description on page <u>3</u> , line <u>30</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution <p style="text-align: center;"><b>CENTRAALBUREAU VOOR SCHIMMELCULTURES</b></p>	
Address of depositary institution (including postal code and country) <p style="text-align: center;"><b>Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, the Netherlands</b></p>	
Date of deposit <p style="text-align: center;"><b>19 October 1995</b></p>	Accession Number <p style="text-align: center;"><b>CBS 704.95</b></p>
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<p>In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).</p>	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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### INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

<b>A. The indications made below relate to the microorganism referred to in the description</b> on page <u>3</u> , line <u>29-30</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution <p style="text-align: center;"><b>CENTRAALBUREAU VOOR SCHIMMELCULTURES</b></p>	
Address of depositary institution (including postal code and country) <p style="text-align: center;"><b>Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, the Netherlands</b></p>	
Date of deposit <p style="text-align: center;"><b>19 October 1995</b></p>	Accession Number <p style="text-align: center;"><b>CBS.703.95</b></p>
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<p style="text-align: center;">In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).</p>	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>3</u> , line <u>29</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution <b>CENTRAALBUREAU VOOR SCHIMMELCULTURES</b>	
Address of depositary institution (including postal code and country)  <b>Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, the Netherlands</b>	
Date of deposit <b>19 October 1995</b>	Accession Number <b>CBS 702.95</b>
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<b>In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).</b>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

<b>A.</b> The indications made below relate to the microorganism referred to in the description on page <u>3</u> , line <u>28-29</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution <p style="text-align: center;"><b>CENTRAALBUREAU VOOR SCHIMMELCULTURES</b></p>	
Address of depositary institution (including postal code and country) <p style="text-align: center;"><b>Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, the Netherlands</b></p>	
Date of deposit <p style="text-align: center;"><b>19 October 1995</b></p>	Accession Number <p style="text-align: center;"><b>CBS 701.95</b></p>
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<p>In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).</p>	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	


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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>3</u> , line <u>27</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution <b>CENTRAALBUREAU VOOR SCHIMMELCULTURES</b>	
Address of depositary institution (including postal code and country)  <b>Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, the Netherlands</b>	
Date of deposit <b>27 December 1994</b>	Accession Number <b>CBS 651.94</b>
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<p>In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).</p>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

<b>A.</b> The indications made below relate to the microorganism referred to in the description on page <u>3</u> , line <u>27-28</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution <p style="text-align: center;"><b>CENTRAALBUREAU VOOR SCHIMMELCULTURES</b></p>	
Address of depositary institution (including postal code and country) <p style="text-align: center;"><b>Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, the Netherlands</b></p>	
Date of deposit <p style="text-align: center;"><b>20 December 1994</b></p>	Accession Number <p style="text-align: center;"><b>CBS 620.94</b></p>
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<p>In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).</p>	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

<b>A.</b> The indications made below relate to the microorganism referred to in the description on page <u>3</u> , line <u>26</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution <p style="text-align: center;"><b>CENTRAALBUREAU VOOR SCHIMMELCULTURES</b></p>	
Address of depositary institution (including postal code and country) <p style="text-align: center;"><b>Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, the Netherlands</b></p>	
Date of deposit <p style="text-align: center;"><b>20 December 1994</b></p>	Accession Number <p style="text-align: center;"><b>CBS 618.94</b></p>
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<p style="text-align: center;">In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).</p>	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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## CLAIMS

1. A transglutaminase preparation, wherein the transglutaminase producing strain belongs to the class  
5 *Oomycetes*.
2. The transglutaminase preparation according to claim 1, wherein the transglutaminase producing strain belongs to the order *Peronosporales*.  
10
3. The transglutaminase preparation according to claim 2, wherein the transglutaminase producing strain belongs to the family *Pythiaceae*.
- 15 4. The transglutaminase preparation according to claim 3, wherein the transglutaminase producing strain belongs to a genus selected from *Pythium* and *Phytophthora*.
- 20 5. The transglutaminase preparation according to claim 4, wherein the transglutaminase producing strain belongs to a species selected from *Pythium* sp., *Pythium irregulare*, *Pythium dissotocum*, *Pythium periillum* (or *P. periplocum*), *Pythium periillum* (or *P. periplocum*), *Pythium torulosum*, *Pythium ultimum* and *Pythium aphanidermatum*, preferably from  
25 the species *Pythium irregulare*, CBS 701.95, *Pythium* sp., CBS 702.95, *Pythium intermedium*, CBS 703.95, *Pythium* sp., CBS 704.95, *Pythium ultimum*, CBS 705.95, or the species *Pythium periillum* (or *P. periplocum*), CBS 620.94.
- 30 6. The transglutaminase preparation according to claim 4, wherein the transglutaminase producing strain belongs to a species selected from *Phytophthora cactorum*, *Phytophthora palmivora*, *Phytophthora porri*, *Phytophthora infestans*, *Phytophthora megasperma*, *Phytophthora cinnamomi* and  
35 *Phytophthora cryptogea*, preferably from the species *Phytophthora cactorum*, CBS 618.94 or IFO 30474, or the species *Phytophthora cryptogea*, CBS 651.94.

7.. The transglutaminase preparation according to claim 2, wherein the transglutaminase producing strain belongs to a family selected from *Peronophytophthoraceae* and *Albuginaceae*.

5

8. The transglutaminase preparation according to claim 2, wherein the transglutaminase producing strain belongs to the family *Peronosporaceae*.

10 9. The transglutaminase preparation according to claim 8, wherein the transglutaminase producing strain belongs to the genus *Plasmopara*.

10. The transglutaminase preparation according to claim 9,  
15 wherein the transglutaminase producing strain belongs to the species *Plasmopara halstedii*.

11. The transglutaminase preparation according to claim 1,  
wherein the transglutaminase producing strain belongs to  
20 the order *Saprolegniales*.

12. The transglutaminase preparation according to claim 11,  
wherein the transglutaminase producing strain belongs to  
the family *Saprolegniaceae*.

25

13. The transglutaminase preparation according to claim 12,  
wherein the transglutaminase producing strain belongs to a  
genus selected from *Achlya*, *Saprolegnia* and *Aphanomyces*.

30 14. The transglutaminase preparation according to claim 11,  
wherein the transglutaminase producing strain belongs to a  
family selected from *Ectrogellaceae*, *Haliphothoraceae* and  
*Leptolegniellaceae*.

35 15. The transglutaminase preparation according to claim 1,  
wherein the transglutaminase producing strain belongs to  
the order *Leptomitales*.

16. The transglutaminase preparation according to claim 15, wherein the transglutaminase producing strain belongs to the family *Leptomitaceae*.
- 5 17. The transglutaminase preparation according to claim 16, wherein the transglutaminase producing strain belongs to a genus selected from *Apodachlya* and *Leptomitus*.
18. The transglutaminase preparation according to claim 15,  
10 wherein the transglutaminase producing strain belongs to the family *Rhiphidiaceae*.
19. The transglutaminase preparation according to claim 18,  
15 wherein the transglutaminase producing strain belongs to a genus selected from *Aqualinderella* and *Rhiphidium*.
20. The transglutaminase preparation according to claim 1,  
20 wherein the transglutaminase producing strain belongs to the order *Lagenidiales*.
21. The transglutaminase preparation according to claim 20,  
wherein the transglutaminase producing strain belongs to the family *Lagenidiaceae*.
- 25 22. The transglutaminase preparation according to claim 21, wherein the transglutaminase producing strain belongs to a genus selected from *Lagenidium* and *Olpidiopsis*.
23. The transglutaminase preparation according to claim 20,  
30 wherein the transglutaminase producing strain belongs to a family selected from *Olpidiaceae* and *Sirolpidiaceae*.
24. A parent transglutaminase derived from or producible by  
35 *Phytophthora cactorum*, CBS 618.94 or IFO 30474,  
*Phytophthora cryptogea*, CBS 651.94, *Pythium periillum* (or *P. periplocum*), CBS 620.94, *Pythium irregulare*, CBS 701.95, *Pythium sp.*, CBS 702.95, *Pythium intermedium*, CBS 703.95, *Pythium sp.*, CBS 704.95, *Pythium ultimum*, CBS 705.95 or a functional analogue of said transglutaminase which

(i) comprises an amino acid sequence being at least 40% homologous with the amino acid sequence of the parent transglutaminase,

5 (ii) reacts with an antibody raised against the parent transglutaminase, and/or

(iii) is encoded by a DNA sequence which hybridizes with the same probe as a DNA sequence encoding the parent  
10 transglutaminase.

25. A method for the production of a transglutaminase preparation comprising cultivation in a suitable nutrient medium a strain selected from the strains *Phytophthora cactorum*, CBS 618.94 or IFO 30474, *Phytophthora cryptogea*, CBS  
15 651.94, *Pythium irregulare*, CBS 701.95, *Pythium sp.*, CBS 702.95, *Pythium intermedium*, CBS 703.95, *Pythium sp.*, CBS 704.95, *Pythium ultimum*, CBS 705.95 and *Pythium perillium* (or *P. periplocum*), CBS 620.94.

20

26. The transglutaminase preparation according to any of the claims 1-23 which further comprises a stabilizer.

27. A DNA construct comprising a DNA sequence encoding an  
25 enzyme exhibiting transglutaminase activity, which DNA sequence comprises

a) the DNA sequence shown in SEQ ID No. 1, and/or the DNA sequence obtainable from the plasmid in *Escherichia coli*  
30 DSM 10256 or

b) an analogue of the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from the plasmid in *Escherichia coli* DSM 10256, which

35

i) is homologous with the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from the plasmid in *Escherichia coli* DSM 10256, or

ii) hybridizes with the same oligonucleotide probe as the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from the plasmid in *Escherichia coli* DSM 10256, or

5

iii) encodes a polypeptide which is homologous with the polypeptide encoded by a DNA sequence comprising the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from the plasmid in *Escherichia coli* DSM 10256,

10 or

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified transglutaminase encoded by the DNA sequence shown in SEQ ID No 1 and/or the DNA sequence obtainable from the plasmid in *Escherichia coli* DSM 10256.

15

28. The DNA construct according to claim 27, in which the DNA sequence encoding an enzyme exhibiting transglutaminase activity is obtainable from a microorganism, preferably a fungus.

29. The DNA construct according to claim 28, in which the DNA sequence is obtainable from a strain of *Pythium* or *Phytophthora*.

25

30. The DNA construct according to claim 29, in which the DNA sequence is isolated from or produced on the basis of a DNA library of a strain of *Phytophthora*, in particular *Phytophthora cactorum*, CBS 618.94.

30

31. A recombinant expression vector comprising a DNA construct according to any of claims 27-30.

32. A cell comprising a DNA construct according to any of claims 27-30 or a recombinant expression vector according to claim 31.

35

33. A cell according to claim 32, which is a eukaryotic



cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell.

34. A cell according to claim 33, wherein the cell belongs  
5 to a strain of *Aspergillus*, in particular a strain of *Aspergillus niger* or *Aspergillus oryzae*.

35. A method of producing an enzyme exhibiting  
transglutaminase activity, the method comprising culturing  
10 a cell according to any of claims 32-34 under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

36. An enzyme exhibiting transglutaminase activity, which  
15 enzyme  
a) is encoded by a DNA construct according to any of claims 27-30,

b) produced by the method according to claim 35, and/or  
20

c) is immunologically reactive with an antibody raised against a purified transglutaminase encoded by the DNA sequence shown in SEQ ID No 1 and being derived from *Phytophthora cactorum*, CBS 618.94.  
25

37. A transglutaminase composition comprising the transglutaminase according to claim 24 or 36 and a stabilizer.

38. A method of crosslinking proteins wherein a transglutaminase preparation according to any of the claims 1-23 or a  
30 transglutaminase according to claim 24 or 36 is contacted with a proteinaceous substrate.

39. Use of the transglutaminase preparation according to  
35 any of the claims 1-23 or the transglutaminase according to claim 24 or 36 in flour, meat products, fish products, cosmetics, cheese, milk products, gelled food products and leather finishing.

40. Use of the transglutaminase preparation according to any of the claims 1-23 or the transglutaminase according to claim 24 or 36 in the production of dough or baked products.

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/10 // (C12N 9/10, C12R1:645)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPOQUE, PAJ, MEDLINE, BIOSIS, DERWENT BIOTECH ABSTRACT, SCISEARCH, PCI, CIENSEQ, SWISSPOT/EMBL/DBJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 8907398 A1 (BONGRAIN S.A.), 24 August 1989 (24.08.89), see claims --	1-40
X	Dialog Information Services, file 357, Derwent Biotechnology Abs, Dialog accession no. 081928, DBA accession no. 88-12777, Tomita K et al: "Distribution and properties of gamma-glutamyl- transpeptidase in filamentous fungi - gamma- glutamyltransferase"; & Agric. Biol. Chem. (52, 9, 2373-74) 1988 --	1-40

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

## \* Special categories of cited documents

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
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\*X\* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Name and mailing address of the ISA/  
Swedish Patent Office  
Box 5055, S-102 42 STOCKHOLM  
Facsimile No. +46 8 666 02 86

Authorized officer

Patrick Andersson  
Telephone No. +46 8 782 25 00

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, file 357, Derwent Biotechnology Abs, Dialog accession no. 100493, DBA accession no. 90-03184, Tomita K et al: "Synthesis of gamma-glutamyltaurine by gamma- glutamyltranspeptidase by <i>Penicillium roqueforti</i> - gamma-glutamyltransferase activity"; & Agric. Biol. chem. (53, 12, 3239-44) 1989  --	1-40
X	EP 0481504 A1 (AMANO PHARMACEUTICAL CO., LTD.), 22 April 1992 (22.04.92), see the whole document especially page 9, line 23-25  -- -----	1-40

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

01/04/96

International application No.

PCT/DK 96/00031

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			FR-A,B-	2627062	18/08/89
			JP-T-	2503148	04/10/90
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EP-A1-	0481504	22/04/92	DE-D-	69116495	00/00/00
			JP-A-	5199883	10/08/93
			US-A-	5420025	30/05/95
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